Factors Associated with Severe Granulomatous Pneumonia in *Mycobacterium tuberculosis*-Infected Mice Vaccinated Therapeutically with hsp65 DNA

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Resistant C57BL/6 mice infected in the lungs with *Mycobacterium tuberculosis* and then therapeutically vaccinated with *Mycobacterium leprae*-derived hsp65 DNA develop severe granulomatous pneumonia and tissue damage. Analysis of cells accumulating in the lungs of these animals revealed substantial increases in T cells secreting tumor necrosis factor alpha and CD8 cells staining positive for granzyme B. Stimulation of lung cells ex vivo revealed very high levels of interleukin-10, some of which was produced by B-1 B cells. This was probably an anti-inflammatory response, since lung pathology was dramatically worsened in B-cell gene-disrupted mice.

The continuing global epidemic caused by *Mycobacterium tuberculosis* has emphasized the need for new vaccines to replace or boost the existing BCG vaccine (11). Among a considerable variety of different types of vaccines (4, 10, 11), those based upon DNA have had some degree of success, including DNA vaccines directed against Ag85 (6) and the fusion protein Mtb72F (13), as well as pools of DNA constructs (3). To date, DNA vaccines in humans have not been very effective, but the potential to use tuberculosis DNA vaccines in prime boost regimens justifies continued evaluation.

An important issue is safety, however. A vaccine based upon the hsp65 molecule of *Mycobacterium leprae* was initially reported to be very effective in a mouse model (8, 14), but this was not confirmed, and when given in a postexposure or therapeutic mode it caused severe necrosis or severe pneumonia in the lungs depending upon the strain of mouse used (15). These lesions were reminiscent of the so-called Koch reaction (12), and probably reflected the expansion of T cells by this highly immunogenic vaccine, which mediated an overexuberant response in the lungs causing tissue damage.

Our earlier studies did not investigate this issue, however, and so we returned to this question in the present study. Several parameters were monitored in the lungs of mice given up to four immunizations with hsp65 DNA. It was found that the lungs of such mice accumulated large numbers of both CD4 and CD8 cells secreting tumor necrosis factor alpha (TNF- α), that there were considerable increases in CD8 cells staining positive for granzyme B, and that cells restimulated ex vivo produced very high levels of the cytokine interleukin-10 (IL-10). These data are consistent with the florid and very extensive granulomatous pneumonia and the increasing tissue dam-

age. In addition, the very high IL-10 production would presumably suppress any positive protective response of the vaccine.

Specific-pathogen-free female BALB/c, C57BL/6, and B-cell-knockout mice, 6 to 8 weeks old, were purchased from the Jackson Laboratories, Bar Harbor, Maine. B-cell-knockout mice were on a C57BL/6 background and lacked mature B cells. Mice were challenged by low-dose aerosol exposure with *M. tuberculosis* strain H37Rv using a Glas-Col (Terre Haute, Ind.) aerosol generator calibrated to deliver 50 to 100 bacteria into the lungs.

The DNA vaccine encoding the hsp65 protein antigen of *M. leprae* was constructed using the plasmid vector pCDNA3 (9, 14). BALB/c and C57BL/6 mice were injected intramuscularly four times at 2-week intervals with 50 μ g hsp65 DNA per quadriceps muscle using a 30-gauge needle and syringe beginning 8 weeks after the aerosol infection with *M. tuberculosis*. As negative controls, mice received injections with control plasmid DNA (pCDNA3) or with saline.

Lungs were harvested for bacterial counts, flow cytometric analysis, and immunohistochemistry 1 week following each vaccination. Bacterial counts in the lungs were determined by plating serial dilutions of homogenates of the right middle lobe on nutrient 7H11 agar and counting CFU following 3 weeks incubation at 37°C. In order to obtain single-cell suspensions, lung digests using collagenase and DNase were performed on the right cranial, accessory, and left lung lobes from each individual mouse as previously described (8). The cells were prepared for intracellular staining by incubation at 37°C with anti-CD3 ϵ (145-2C11; 0.1 μ g/ml), anti-CD28 (clone 37.51; 1 μ g/ml), and monensin (3 μ M; Fix/Perm kit, PharMingen) for 4 h.

Cells were stained with peridinin chlorophyll protein-anti-CD8a (clone 53-6.7) and allophycocyanin-anti-CD4 (clone RM4-5) before the permeabilization step according to the kit instructions. Fluorescein isothiocyanate-anti-TNF- α (clone

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A.

B.

C.

FIG. 1. Hsp65 DNA vaccination induced an increase of (A) TNF- α and (B) granzyme B in the lungs, but not (C) IFN- γ . Lung cells from hsp65 DNA-vaccinated mice previously infected by aerosol *M. tuberculosis* were incubated with fluorescently labeled antibodies for surface markers CD4, CD8, and intracellular TNF- α , IFN- γ , and granzyme B. Data are expressed as the mean percentage of cells from five mice per group. Open squares, vector control-treated mice; solid squares, hsp65-treated mice. Error bars represent $\pm$ standard error of the mean. *, $P < 0.05$; **, $P < 0.001$ compared to the vector control group using Student's t test.

MP6-XT22), fluorescein isothiocyanate-isotype control antibody (rat IgG1), phycoerythrin-anti-gamma interferon (IFN- γ) (clone XMG1.2), phycoerythrin -isotype control antibody (rat IgG2b), allophycocyanin-anti-granzyme B (clone GB12), and allophycocyanin-isotype control antibody (mouse immuno-

globulin G1) were incubated with the appropriate surface-stained cells for 30 min, and the cells were washed twice and resuspended in DRPMI prior to analysis. All antibodies were purchased from BD PharMingen unless otherwise stated, and were used at 25 μ g/ml.

FIG. 2. Hsp65 DNA vaccine induced an increase in IL-10 production in the lungs. Lung cells isolated from hsp65 DNA-vaccinated mice previously infected with *M. tuberculosis* were incubated with culture filtrate protein at 37°C and frozen after 72 h. Supernatants from five mice per group were pooled, and a cytometric bead array was run to detect secreted IL-10 protein. Open squares, vector control-treated mice; solid squares, hsp65-treated mice.

The cells stained for granzyme B were previously incubated overnight with 2 μ g/ml *M. tuberculosis* culture filtrate protein at 37°C. Cells were gated on lymphocytes by forward scatter and side scatter according to their characteristic scatter profile

which is small size and low granularity. Individual cell populations were identified according to the presence of specific fluorescent-labeled antibody, and all analyses were performed with an acquisition of at least 100,000 events on a Becton Dickinson FACScalibur flow cytometer. A Cytometric Bead Array kit (BD Biosciences, San Jose, CA) was used to measure IL-10 in the supernatant of lung cell suspensions incubated for 72 h at 37°C with culture filtrate protein at 2 μ g/ml and then frozen back at -80°C . After thawing, the cytometric bead array mouse inflammation assay procedure was performed according to kit instructions, and the beads were analyzed on the FACScalibur flow cytometer. The sensitivity range for IL-10 according to the cytometric bead array kit specifications was 17.5 pg/ml.

Whole lungs were prepared and sectioned for immunohistochemistry as described previously (7). Tissue sections were incubated overnight at 4°C with purified primary antibodies from BD PharMingen at appropriate concentrations against CD8a (clone 53-6.7) and B220 (clone RA3-6B2). Other sections were incubated with isotype control rat immunoglobulin G2a. After washing, all sections were incubated with the secondary detection antibody goat F(ab')₂ anti-rat immunoglobulin conjugated to horseradish peroxidase (BioSource, Camarillo, CA), and the reaction was developed using amin-oethylcarbazole (BioGenex, San Ramon, CA) as substrate. Sections were counterstained with Meyer's hematoxylin.

Lungs were harvested for viable bacteria counts, but the

FIG. 3. Representative photomicrographs of lungs from *M. tuberculosis*-infected B-cell-knockout mice 68 days after the last of four vaccinations with hsp65 DNA. Panel A, mouse did not receive a vaccine. Panels B and C, mice received four intramuscular vaccinations with hsp65 DNA. Panel A shows a moderate, multifocal granulomatous pneumonia. Panel B shows a severe, diffuse, fibrinous, necrotizing granulomatous pneumonia. Panel C shows that in some cases, the lumens of large airways are plugged with fibrin and necrotic cellular debris (*). Multiple foci of neutrophils and eosinophils (arrows) were visibly associated with areas of necrosis. The middle right lung lobe was inflated with 10% formalin, processed and sectioned routinely, and stained with hematoxylin and eosin. A and B: 10 $\times$ total magnification. C: 40 $\times$ total magnification.

FIG. 4. Representative photomicrographs showing immunohistochemical staining for CD8⁺ (left panels) and B220-positive (right panels) T cells within saline controls (A and B) and mice vaccinated four times with hsp65 (C and D). Well-organized lung lesions from C56BL/6 mice consisting of both macrophages and lymphocytes developed through 90 days postinfection. Arrow bars depict centralized aggregates of CD8⁺ T cells (A). Lung lesions from hsp65-vaccinated mice were larger and showed tissue necrosis depicted by parenchymal thickening and cholesterol deposition (photomicrograph not shown). Mice vaccinated with hsp65 showed disorganized lesions with scattered foci of CD8⁺ T cells (C). Peribronchial and perivascular cuffing of lymphocytes from saline controls showed moderate numbers of lymphocytes with aggregates of B220-positive cells depicted by arrow bars (B), while hsp65-vaccinated mice showed increased numbers of lymphocyte aggregates consisting of more B220-positive cells (D). A to D: 10 $\times$ total magnification.

hsp65 vaccine was not protective as no differences in bacterial counts in the lungs were observed between the hsp65 DNA-vaccinated, saline-treated, or vector-treated groups (data not shown). Lung cells were isolated from mice after each round of vaccinations and analyzed for cytokine and granzyme B expression. As shown in Fig. 1A, there was a substantial increase in both CD4 and CD8 cells staining positive for TNF- α in the lungs of infected mice vaccinated with the hsp65 DNA compared to mice treated with the control vector. Likewise, staining for granzyme B revealed an increase of CD8 cells positive for this molecule (Fig. 1B). In contrast, although there was a slight increase in the percentage of IFN- γ -producing CD4 T cells in the vector-treated mice after the fourth injection, there were no overt differences in the numbers of CD8 T cells staining positive for IFN- γ in the vaccinated and vector control groups (Fig. 1C). There were no significant differences in these T cell phenotypes between the vector-treated and saline-treated groups (data not shown).

Lung cells were also analyzed for inflammatory cytokines using a cytometric bead assay. It was found that cells from mice given the hsp65 DNA vaccine had very high levels of IL-10

secretion compared to the vector group (Fig. 2). No differences in IL-10 secretion were observed between the vector and saline control groups (data not shown). This cytokine can be made by a variety of sources, particularly macrophages and B-1 B cells, and so we performed a preliminary experiment using B-cell-knockout mice. In such animals, lung pathology was considerably worsened in the B-cell-knockout mice treated with hsp65 DNA compared to B-cell-knockout mice treated with saline, as there was massive consolidation of the lung tissue and airway blockage in the hsp65 DNA-treated B-cell-knockout mice (Fig. 3B and 3C) compared to the saline-treated control B-cell-knockout mice- which showed only moderate, multifocal granulomatous pneumonia (Fig. 3A). The vector control DNA appeared to have no histologic effect as lung tissue sections from mice treated with saline were similar to the vector-treated mice (data not shown). These results suggest that secretion of IL-10 in the lungs of the hsp65-vaccinated mice was at least in part a host protective physiological response aimed at dampening the huge granulomatous cell influx.

In addition, in order to determine if the hsp65 DNA-treated mice contained more B cells as potential producers of the

increased IL-10 in the lung than saline-treated control mice, lung tissue sections were stained for the B220 marker. Immunohistochemical staining of lung tissue sections showed an increase the number of B220-positive cells in the lungs of hsp65 DNA-treated mice compared to saline controls (Figs. 4B and 4D). Immunohistochemical staining of lung tissue sections also showed that while lung sections from the saline-treated mice showed well-organized granulomas composed of macrophages and lymphocytes, including centralized regions of CD8⁺ T cells (Fig. 4A), lung lesions in the mice treated with the hsp65 vaccine were disorganized with scattered foci of CD8⁺ T cells (Fig. 4C). No differences in lymphocyte staining were observed between the saline and vector controls.

These observations thus suggest that therapeutic vaccination of mice already infected with *M. tuberculosis* with the hsp65 DNA of *M. leprae* creates an exuberant response that promotes a huge, disorganized granulomatous response leading to consolidation of the lung tissues. This is almost certainly driven by CD4 and CD8 T cells secreting TNF- α , a cytokine that strongly promotes granuloma formation (1), but which can be tissue damaging itself at high concentrations (2). Moreover, although the C57BL/6 mouse is more resistant to necrosis than our previous observations in the BALB/c strain (15), the increased influx of CD8 cells positive for granzyme B could also potentially contribute to increasing tissue damage. As an attempt to reverse this, we speculate that the host triggers secretion of very high amounts of IL-10 to try to counteract this, an idea that is supported by our intriguing observation that the lung pathology becomes even worse in mice lacking B cells, a source of this cytokine.

In addition, the accumulation of cells capable of secreting molecules that directly or indirectly result in inflammation and damage was shown here to be dependent on the frequency of inoculation, as we have previously shown that no adverse effects were seen until the mice received at least three injections of the hsp65 DNA (15). If a DNA vaccine could be identified that had a strong protective therapeutic effect after a single injection, then it could potentially avoid the safety issues identified above.

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